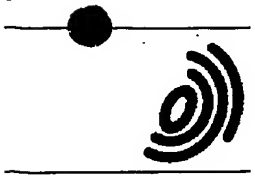

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

03255200.2

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Polysialic acid derivatives

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

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POLYSIALIC ACID DERIVATIVES

The present invention relates to polysialic acid derivatives which are useful for conjugation to proteins and peptides, or to drug deilvery systems such as liposomes, having sulfhydryl groups.

The extended presence of drugs either within the vascular system or in extravascular use is often a pre-requisite for their optimal use. Many antibiotics and cytostatics for instance, as well as a variety of therapeutic peptides in proteins, and liposomes are removed from the circulation prematurely and before effective concentrations in target tissues can be achieved. The half lives of a number of short-lived proteins (for instance enzymes, cytokines, etc) have been augmented by conjugating these to low poly(ethylene glycol). It appears that PEG molecules prolong the circulation time of proteins and particles by forming a cloud around their surface, thus sterically hindering interaction with factors responsible for their clearance. However PEG is non-biodegradable and accumulation of PEGylated proteins intracelluiary may be undesirable especially on chronic use.

We have described the conjugation of a polysaccharide comprising 2→8 and or 2→9 (e.g. alternating 2→8 and 2→9) linked sialic acid units conjugated to proteins to increase their half life, reduce their immunogenicity/antigenicity or increase the stability of a variety of proteins. In WO92/22331, polysialic acids are reacted with a model drug and shown to extend the half life in the circulation of mice. In Cell. Mol. Life Sci. 57 (2000) 1964 to 1969 and in Biotechnol. Genet. Eng. Rev. 16 (1999) 203 to 215, Gregoriadis *et al* describe the conjugation of polysialic acids to asparaginase and catalase, and show that the clearance rates from circulation reduced whilst enzyme activity was retained. We have also polysialylated insulin (Biochim. Biophys. Acta 1622 (2003) 42-49 and shown it to be active. We have also polysialylated interferon (AAPS Annual meeting 2002, Toronto, Canada, M1056). We have also polysialylated antibody fragment Fab (Epenetos, A. *et al.*, Proceedings of ASCO (Clinical Pharmacy) 21 (2002) 2186).

In all of these publications, polysialic acid is rendered reactive, by generating an aldehyde group at the non-reducing end by oxidation of the 7, 8-vicinaldiol moiety with sodium periodate. The aldehyde group was then reacted with primary amine groups on proteins, generally assumed to be epsilon-amino groups of lysine moieties of the protein or N-terminal amine groups. The reaction forms a Schiff base which is reduced by cyanoborohydride to a secondary amine.

In WO-A-01/87922 we also suggest that derivatisation with other molecules could be carried out in the presence of denaturant to achieve increased levels of derivatisation. Examples of other derivatising agents are polyethylene glycol compounds. Activated PEG compounds such as tresyl-PEG and succinimidyl succinate ester of PEG were mentioned. The examples used succinimidyl succinate activated PEG, which is believed to react with amine groups. We suggested that PEG compounds could be made reactive with hydroxyl or thiol groups.

PEG derivatives having functional groups for coupling to thiol groups are commercially available. The functional groups may be maleimide, vinyl sulfone, iodoacetamide or orthopyridyl disulphide groups. Since these reagents react specifically with cysteines, and since proteins have fewer cysteines on their surfaces than lysine groups, the derivatisation is more controllable. Furthermore, in the absence of a free cysteine in a native protein, one or more free cysteines may be added by genetic engineering. The advantage of this approach is that it makes possible site-specific derivatisation at areas on the protein which will minimise a loss in biological activity.

It would be useful for modification by polysialic acid to be targeted towards thiol (sulfhydryl) groups. It would also be desirable for the efficiency of derivatisation by sialic acid to be increased, the processes described in our prior art mentioned above requiring high excesses of active polysialic acid. It would also be desirable to avoid the use of cyano borohydride.

According to the present invention there is provided a novel compound comprising a polysialic acid having a moiety linked at one or each terminal unit which includes a functional group selected from N-maleimido groups vinysulphone gruops, N-iodoacetamide groups and orthopyridyl disulphide groups.

The terminal unit to which the moiety is linked may be at the non-reducing end of the polysialic acid or at the reducing end of the polysialic acid. Generally the terminal sialic acid unit has been subjected to a preliminary chemical reaction to generate useful functional groups to which a maleimide-group containing reagent may be linked. We have found it convenient to use the chemistry disclosed in our earlier publications in which an aldehyde group is generated, as a preliminary step to generate the functional group via which the maleimide moiety may be linked.

In our earlier publications mentioned above, it is the non-reducing terminal unit which is converted into an aldehyde moiety by oxidation of the 7, 8-vicinal diol moiety with sodium periodate to form the carbon 7-aldehyde compound. This is an appropriate reaction for the present invention.

As an alternative, it is possible to provide the aldehyde moiety at the reducing terminal unit. In this case, a preliminary step in which the non-reducing end is deactivated, by preliminary oxidation and reduction steps, is carried out. The reduction step converts the ketal unit at the reducing end into a reduced ring opened form, having vicinal diols. The vicinal diols are subsequently oxidised using sodium periodate to form an aldehyde moiety at the carbon atom previously forming the 7-carbon of the reducing terminal unit.

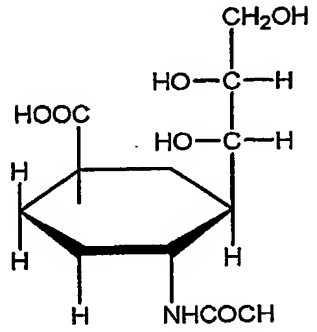
In the invention, the moiety which includes the functional group, may be linked directly to the polysialic acid unit or, more conveniently, may comprise a difunctional organic group such as an alkane diyl group, an arylene group or an oligo(alkyleneoxy) alkane group, or alternatively an oligo peptidyl group. The linkage to the polysialic acid may be a secondary amine, a hydrazone, an alkyl hydrazide an ester or a peptidyl group. The

moiety is generated by reaction of a polysialic acid substrate with a heterobifunctional reagent.

Reagents useful for introducing the selected functional groups are commercially available. A compound which will introduce a maleimide group onto an amine moiety without introducing any additional linker moiety is N-methoxy-carbonyl-maleimide. Generally the reagents include a second functional group for reaction with a group on the polysialic acid which may be the aldehyde group described above, or an amine, hydroxyl N carboxylate group. Suitable second functional groups include N-hydroxy succinimide esters and their sulfosuccimide analogues and hdyrazides. A compound which may be reacted with an aldehyde group, and which includes a C_2-alkanediyl moiety and introduces an –maleimide group is N-[β-maleimidopropionic acid] hydrazide. The hydrazide group reacts with the aldehyde to form a stable hydrazone group. A suitable heterobifunctional compound which includes an oligo(ethyleneoxy) ethylene group is a compound comprising a polyethylene glycol (poly(ethyelenoxy)) group with, at one end, N-hydroxy succinimide (NHS) group and at the other end the functional group. The NHS group reacts with amine groups to form stable amide linkages. Heterobifunctional polyethyleneglycols with NHS at one end and either vinylsulphone or maleimide at the other end are available. Other examples of heterobifunctional reagents include, 3-(2-pyridyldithio)propionyl hydrazide, N-succinimidyl-3-[2-pyridyldithio]propionate, succinimidyl-H-[N-maleimidomethyl]cyclohexane-1-carboxylate), m-maleimidobenzoyl-N-hydroxysuccinimide ester, N-succinimidyl-[4-iodoacetyl] amino benzoate, N-[gamma-maleimidobutyryloxy]succinimide ester, N-[epsilon-maleimidocaproyloxy]succinimide ester and N-succinimidyl iodoacetate. Other reagents are available from Pierce Biotechnology and Shearwater Corporation (polyethylene glycol-based).

Sialic acids (also known as nonulosonic acids) are members of a family of amino containing sugars containing 9 or more carbon atoms. The

most important of the sialic acids is N-acetylneuraminic acid (also known as 5-(acetylamino)-3,5-dideoxy-D-glycero-D-galacto-nonulosonic, lactaminic acid and O-sialic acid) which has the formula:



Polysialic acids may be linked 2→8 and/or 2→9, usually in the α-configuration. In some polysialic acids the linkages are alternating 2→8 and 2→9.

Polysialic acids are generally found to be non-toxic and substantially non-immunogenic. Furthermore the biodegration units, sialic acid, is not known to be toxic and, indeed sialic acids are widely found in animal proteins and cells, including blood cells and circulating proteins.

Polysaccharide compounds containing many sialic acid units are polysaccharides produced by *E. coli*, *Moraxella nonliquifaciens*, *Pasteurella aeroginosis* and *Neisseria meningitidis* or derivatives thereof. For instance colominic acid derived (by hydrolysis to shorten the chain lengths) from *E. coli* KI comprises 2→8 α linked sialic acid units. Polysaccharide from *E. coli* K92 strain comprises alternating 2→8 and 2→9 linked sialic acid units. Polysaccharide C of *N. meningitidis* group C has 2→9 linked sialic acid units.

One group of polysaccharide compounds which has been found to be of particular utility in the invention is group B polysaccharides. These compounds are produced by *Neisseria meningitidis*, *moraxella nonliquifaciens*, *Pasteurella aeroginosis* A2 and *E. coli* K1. These compounds comprise a polysaccharide component comprising sialic acid residues and a phospholipid component. The sialic acid residues are linked

(2 → 8)-α, the naturally occurring polymer consisting of about 200 residues. Some of the glycolipid molecules, especially the high molecular weight compounds appear to have a covalently attached phospholipid at the reducing end of the polysaccharide component.

It is preferable for the bacteria from which the polysaccharide compound is derived to be non-pathogenic for convenience during production. It is particularly suitable therefore for the polysaccharide to be derived from a non-pathogenic strain of *E. coli* such as *E. coli* K92 or,preferably K1 which is non-immunogenic. *E. coli* K92 and K1 isolates are well-known and any such type of any such strains can be used as sources of suitable polysaccharide. In general the polysialic acid should have at least 2, preferably at least 5, more preferably at least 10, for instance more than 50 sialic acid units.

According to the invention, there is also provided a conjugate of a protein and the novel activated polysialic acid. The novel compound comprises a protein with at least one cysteine unit and, linked through a thioether bond to the side chain of a cysteine unit, a polysialic acid through a moiety joined at one or both terminal unit of the polysialic acid.

Where the polysialic acid derivative was a N-maleimido group the moiety will include a N-succinimidyl group, with the thioester linked at the α-carbon atom. Preferably the moiety also comprises a secondary amine, a hydrazone or an amide bond.

There is also provided in the invention a new process in which a polysialic acid is reacted with a heterobifunctional reagent having a first functional group for reaction with sulfhydryl groups and a second functional group different to the first group whereby the said second functional group reacts with a terminal unit of the polysialic acid to form a covalent bond therewith and form a capable of reaction with a sulfhydryl group functional polysialic acid.

In one embodiment the second functional group is a nucleophilic group, preferably hydrazine. This is of particular utility where the polysialic

acid comprises an aldehyde group in the terminal unit whose carbonyl group is attacked by the nucleophilic group.

In another embodiment of the process the second functional group is electrophilic such as an N-alkoxyl carbonyl-imide such as N-hydroxysuccinimide ester or sulphosuccinimide ester, or carbodiimide. The terminal group in such cases is preferably nucleophilic such as amine.

In the process it is preferred that the reagent comprises a bifunctional organic group linking the first and second functional groups. Preferably the bifunctional organic group is selected from a C_{2-18}-alkanediyl group, an arylene group, an oligo peptide and an oligo(alkoxy)alkyl group.

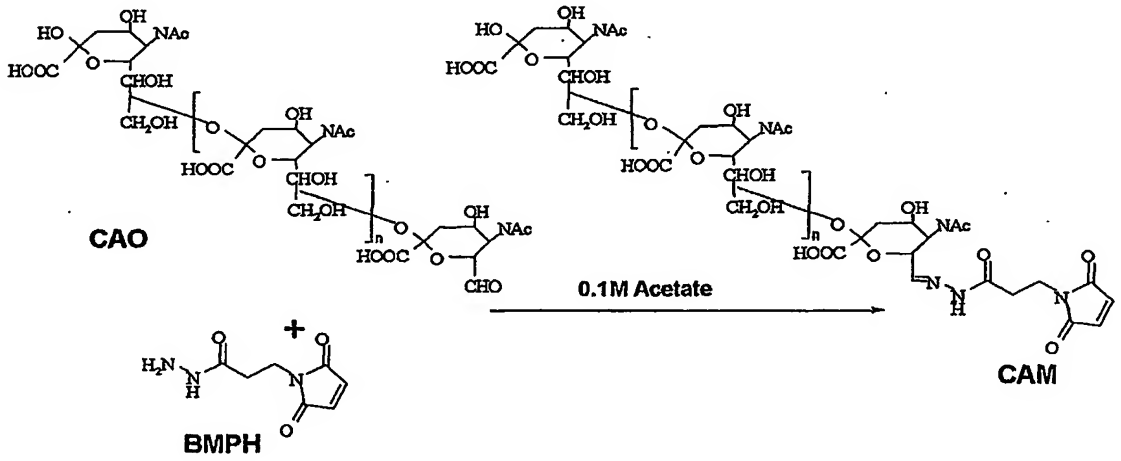
Examples of suitable reagents are given above.

Most usefully the process involves a subsequent step in which the functional polysialic acid is reacted with a polypeptide or a protein having at least one free and unprotected Cys unit whereby the functional group forms a thioether linkage with the thiol group of a Cys unit to form a polysialyated polypeptide or protein. The process is particularly suitable where the protein contains a single Cys unit, whereby site-controlled derivatisation is achieved.

The invention is illustrated in the accompanying examples.

Example 1

1.0 Synthesis Route 1



1.1 Synthesis

Three separate preparations were carried out as follows:

Colominic acid aldehyde (CAO) produced according to WO-A-9222331 (100 mg, 4.4 x10^{-6} mol) was dissolved into 500 µl 0.1 M sodium acetate, to this 5 molar equivalents of *N*-[ß-Maleimidopropionic acid] hydrazide (6.5 mg; 2.2 x10^{-5} mol) was added. This mixture was then vortex mixed and wrapped in foil and allowed to incubate at 37°C for 2h on a rotary mixer. The polymer was then precipitated by the addition of 2 volumes (1.0 ml) of ethanol. The precipitate was collected by centrifugation (13,000 rpm 2 min) in a bench top microcentrifuge. The supernatant was discarded and the pellet dissolved in 500 µml 0.1 M acetate. This process was repeated a further 2 times and the final pellet dissolved in deionised water and freeze dried overnight.

1.2 Assay for Maleimide Content

In this assay cysteine is reacted with the maleimide on the polymer preventing further reaction with Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid)) which contains a disulphide which forms an intense yellow colour when it is substituted for a thiol not adjacent to an aromatic ring. Thus maleimide content can be calculated by measuring the inhition of reaction between cysteine and Ellman's reagent assay.

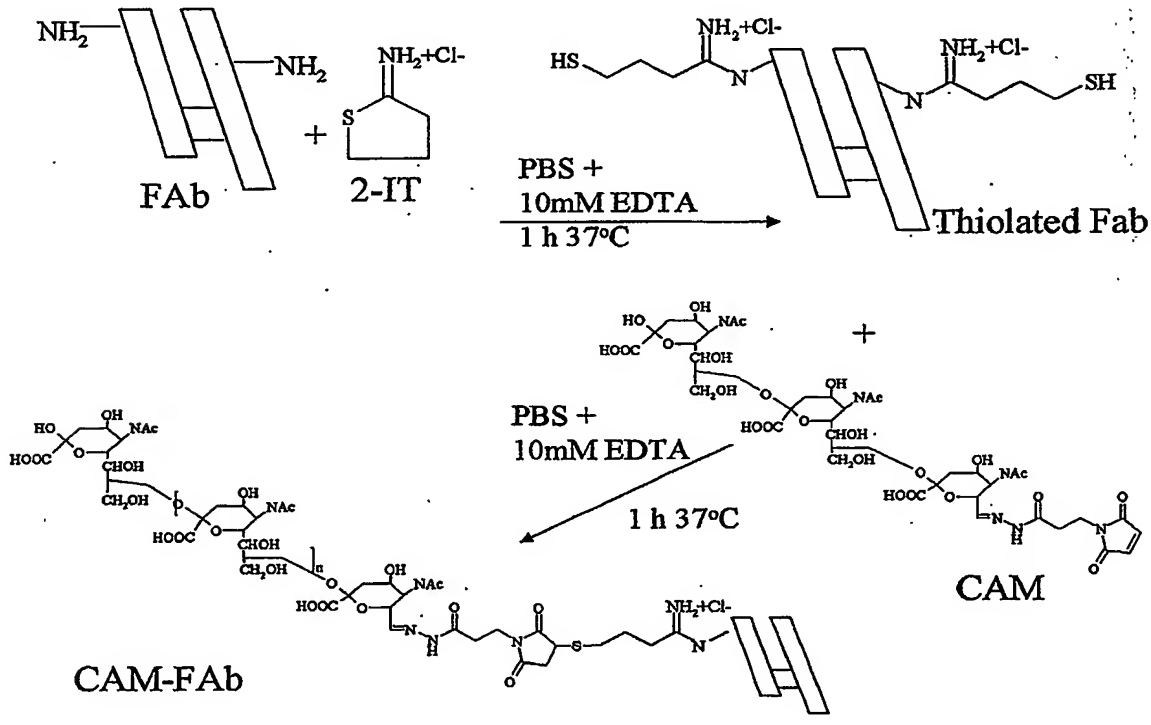
First an initial stock of cysteine at 12 x10^{-3} M (0.145 mg/ml) was prepared in PBS. In a clean microtitre plate 100 µl volume doubling dilutions from 12 x10^{-3} M to 0.375 x10^{-3} M were made from row B to row H. In row A 100 µl PBS was used as a zero standard. Samples of CA and CA-maleimide (CAM) were prepared at 5 or 10 mg/ml and 100 ul of each sample added to duplicate columns of the cysteine dilutions. In one set 100ul PBS without any CA was added as a control. The plate was covered and allowed to incubate at 37°C for 1 h. After this time 20 µl of Ellman's reagent (4 mg/ml in PBS) was added to each well and the plate incubated in the dark at room temperature for 15 min. Absorbance was the measured in wells at 405 nm. Standard curves were then plotted for the samples and the amount of

maleimide present calculated from inhibition of the signal. The results are in Table 1.

1.3 Table 1 - Calculation of Maleimide content:

	r1 5 mg/ml	r2 10 mg/ml	r2 6 mg/ml	r2 10 mg/ml	r3 5 mg/ml	r3 10 mg/ml
Difference from Standards	1.385	1.8695	1.355	1.849	1.297	2.13
less bkg	1.1727	1.6572	1.1427	1.6235	1.0715	1.9045
mole cysteine conj	3.627281163	5.125889267	3.534488092	4.936150806	3.257829127	5.790513834
g/ CAM	0.00082546	0.001166499	0.000804343	0.00112332	0.000741384	0.001317747
g/ml CAM	0.004127302	0.011664986	0.004021717	0.005616599	0.003706921	0.006588736
% of cam in sample	82.54603743	116.649862	80.4343455	112.3319839	74.13841745	131.7747233
average	99.59794974		96.38316469		102.9565704	
Average	99.64 mol %					

1.4 Thiolation of FAb and conjugation to CAM



In the first step a thiol group is introduced into a model protein by thiolation of an amine of lysine.

Ovine FAb (anti Desipramine /Norityrptaline, 4mg, 7.2 x10 $^{-8}$mol) was dissolved in 0.25 ml PBS + 10 mM EDTA to this was added 0.498 mg 2-iminothiolane (2-IT, Traut's reagent 50 mol equiv 3.6 x10^{-6} mol) in 0.25 ml of the same buffer. The tube was wrapped in foil and left to incubate stirring end over end for 1 h at 37°C. Thiolated Fab was purified from free 2-IT by gel filtration (PD-10) and 0.5 ml fractions assayed for presence of protein (BCA assay) or thiol (Ellman's assay). The first eluting peak containing both was pooled and protein and thiols quantified.

1.5 Conjugation of Fab-thiol to CAM

To thiolated FAb (3.6 mg, 6.6x10^{-8} mol) in 2 ml PBS/EDTA 22.5 mg CAM was added (9. x10-7 mol, 15 molar equiv). The tube was sealed and allowed to incubate at 37°C for 1 h whilst gently mixing. The resulting conjugate was then purified according to accepted protocols to remove free CAM. Both CA and protein content were assayed on the conjugate to calculate conjugation ratio.

Control reactions were carried out with CA as a negative control.

Several batches of CAM-Fab were prepared with various degrees of thiolation but maintaining the 15:1 ratio of CAM: Fab. Results are shown in table 2 below:

Table 2

Thiol per FAb	Conjugate ratio (CA: Fab)
1	0.53:1
2	0.9:1
5 (triplicate reaction)	1.925:1 +/- 0.19 : 1 Fab
10	3.51:1

Figure 1 shows an SDS-PAGE gel of triplicate samples and relevant controls

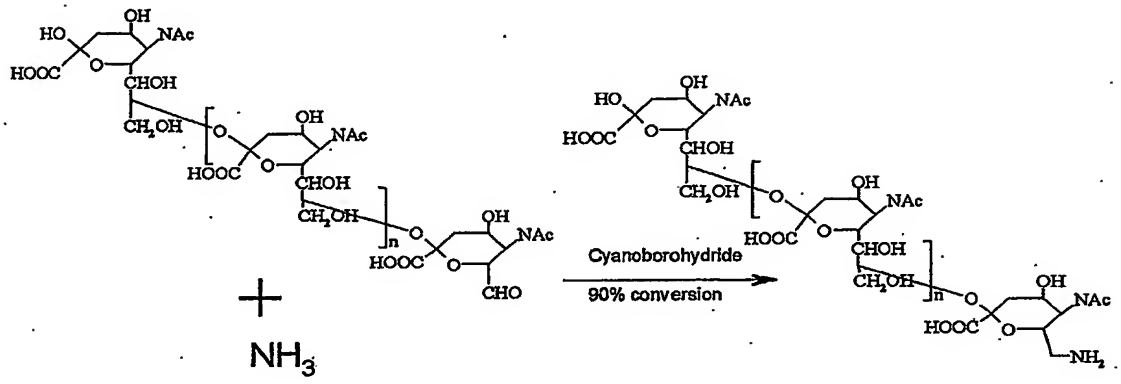
1.6 Conclusion

The results show that in all control wells (samples of thiolated FAb) the migration of the sample is similar to that for fresh FAb (below that of the 50 kDa marker) indicating no cross linking of FAb molecules during the process of conjugation. In the replicate lanes there is considerable band

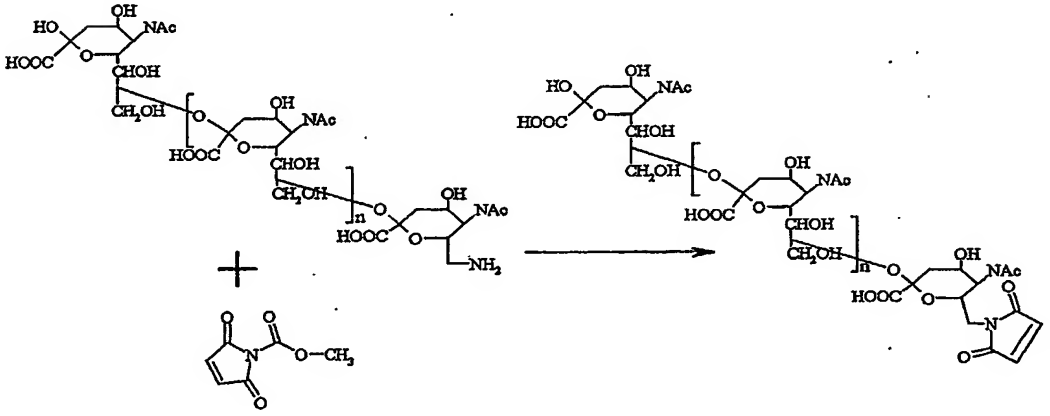
broading with maximum intensity between the 98 and 250 KDa markers which typically indicates an increase in mass which is indicative of a of polysialylated product.

Example 2

Synthesis Route 2



Step 1 amination of CA-aldehyde (CHO)



Step 2 introduction of maleimide ring

Synthesis

2.1. Step 1 amination of oxidised CA

Oxidised colominic acid at (CAO) 10-100 mg/ml was dissolved in 2 ml of deionised water with a 300-fold molar excess of NH_4Cl, in a 50 ml tube and then $NaCNBH_4$ (5 M stock in 1 N NaOH(aq) was added at a final concentration of 5 mg/ml. The mixture was incubated at room temperature for 5 days. A control reaction was also set up with colominic acid (A) instead of CAO. Product colominic acid amine derivative was precipitated by the addition of 5 ml ice-cold ethanol. The precipitate was recovered by centrifugation at 4000 rpm, 30 minutes, room temperature in a benchtop centrifuge. The pellet was retained and resuspended in 2 ml of deionised water, then precipitated again with 5 ml of ice-cold ethanol in a 10 ml ultracentrifuge tube. The precipitate was collected by centrifugation at 30000 rpm for 30 minutes at room temperature. The pellet was again resuspended in 2 ml of deionised water and freeze-dried.

2.2. Assay for amine content

The TNBS (picrylsulphonic acid or 2, 4, 6-tri-nitro-benzene sulphonic Acid) assay was used to determine the amount of amino groups present in the product.

In the well of a microtitre plate TNBS (0.5 µl of 15 mM TNBS) was added to 90 µl of 0.1 M borate buffer pH 9.5. To this was added 10 µl of a 50 mg/ml solution of CA-amide the plate was allowed to stand for 20 minutes at room temperature, before reading the absorbance at 405nm. Glycine was used as a standard, at a concentration range of 0.1 to 1mM. TNBS trinitrophenylates primary amine groups. The TNP adduct of the amine is detected.

Testing the product purified with a double cold-ethanol precipitation using the TNBS assay showed close to 90 % conversion.

2.3. Maleimidation of CA-amine

CA-Amine (17 mg) was dissolved in 1ml deionised water, to this was added 6 mg methoxy-carbonyl-maleimide (MCM). The mixture was left to

react at room temperature for 30 min. To the sample 1 100 µl water and 200 µl acetonitrile was added and then incubated at room temperature for 4 h, after which 300 µl $CHCl_3$ was added the tube shaken and the aqueous fraction collected. Then the fraction was purified over a PD-10 column to remove small molecules. The eluate was freeze dried and assayed for maleimide content. The molar concentration of maleimido was 44 mol %.

CLAIMS

1. A compound comprising a polysialic acid having a pendant moiety linked at one or both terminal unit which includes a functional group selected from N-maleimide groups, vinylsulphone groups, N-iodoacetamide groups orthopyridyl disulphide groups.

2. A compound according to claim 1 in which the pendant moiety is linked at the reducing terminal unit of the polysialic acid.

3. A compound according to claim 1 or claim 2 in which the moiety is linked at the non-reducing terminal unit of the polysialic acid.

4. A compound according to any preceding claim in which the moiety comprises an alkanediyl group and/or an arylene group and a linkage which is a secondary amine linkage, a hydrazone, an alkyl hydrazide linkage or a peptide linkage.

5. A compound according to any preceding claim in which the functional gruop is N-maleimido.

6. A compound comprising a protein with at least one free cysteine unit and, linked through a thioester bond to the side chain of the cysteine unit, with a polysialic acid, through a moiety joined at one or each terminal units of the polysialic acid.

7. A compound according to any preceding claim in which the polysialic acid has at least 2, preferably at least 10, more preferably at least 50 sialic acid units, 2,8 and/or 2,9 linked to one another.

8. A process in which a polysialic acid is reacted with a heterobifunctional reagent having a first functional group selected from N-maleimido groups, vinylsulphone groups, N-iodoacetamide groups orthopyridyl disulphide groups and a second functional group different from the first group whereby the said second functional group reacts with a terminal unit of the polysialic acid to form a covalent bond therewith and form a functional polysialic acid.

9. A process according to claim 8 in which the said second functional group is a nucleophilic group, preferably hydrazine.

10. A process according to claim 9 in which the terminal unit of the polysialic acid has a carbonyl group which reacts with said nucleophilic group.

11. A process according to claim 8 in which the said second functional group is an electrophilic group, preferably an N-alkoxycarbonyl-imide or carbodiimide moiety.

12. A process according to claim 11 in which the terminal unit of the polysialic acid has an amine group which reacts with said electrophilic group, preferably to form a peptide or a urethane linkage.

13. A process according to any of claims 8 to 12 in which the reagent comprises a bifunctional organic group linking the first and second functional groups.

14. A process according to claim 13 in which the bifunctional organic group is selected from a C_{2-18}-alkanediyl group, an arylene group, an oligo peptide and an oligo(alkoxy)alkyl group.

15. A process according to any of claims 8 to 14 in which the first functional group is a N-maleimide group.

16. A process according to any of claims 8 to 15 in which the polysialic acid has at least 2, preferably at least 10, more preferably at least 50, sialic acid units, preferably 2→8 and 2→9 linked to one another.

17. A process according to any one of claims 8 to 16 in which the maleimido-functional polysialic acid is reacted with a polypeptide or a protein having at least one free unprotected Cys unit whereby the maleimide group forms a thioether linkage with the thiol group of a Cys unit to form a polysialyated polypeptide or protein.

18. A process in which a compound according to any of claims 1 to 5 is reacted with a polypeptide or a protein having at least one free and unprotected Cys unit whereby the maleimide group forms a thioether linage with the thiol group of a Cys unit to form a polysialylated polypeptide or protein.

ABSTRACT

POLYSIALIC ACID DERIVATIVES

A polysialic acid compound is reacted with a hetero-bifunctional reagent to introduce a pendant functional group for site-specific conjugation to sulfhydryl groups, for instance side chains of cysteine units in proteins or peptides. The functional group is, for instance, an N-maleimide group.

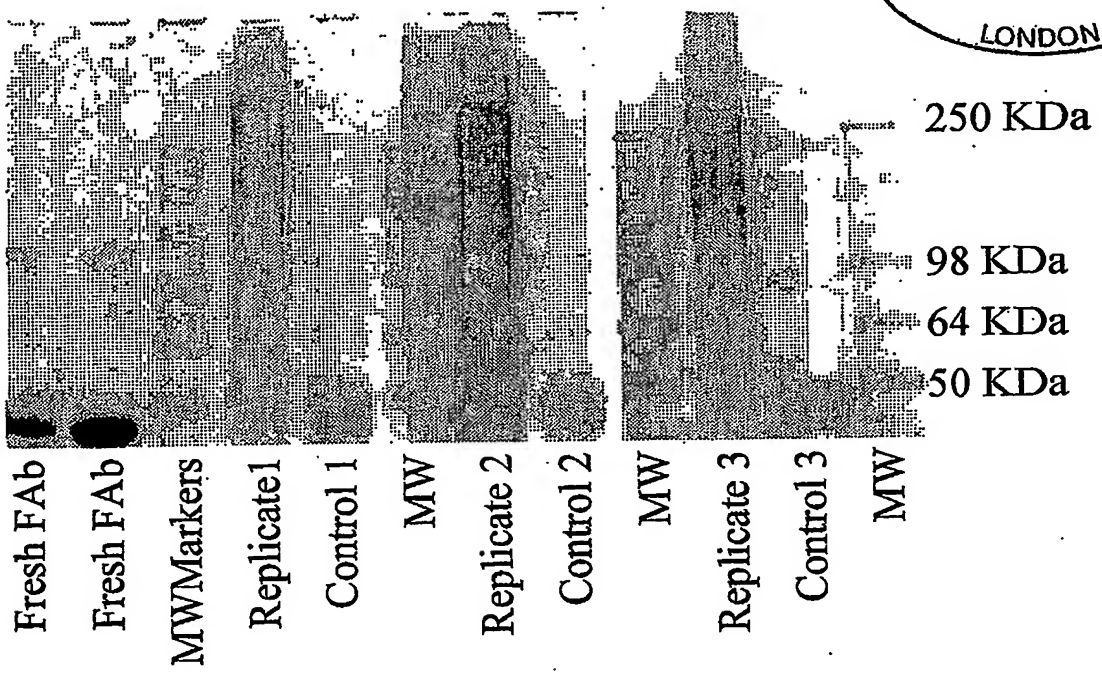


Figure 1